CONINE, MICHAEL B., M.S. Analytical Development for Analysis of American Ginseng and Multiple Agriculturual Chemicals from Soil. (2013)
Directed by Dr. Nadja B. Cech pp. 55

PART I:

Ginseng has been used for thousands of years as an herbal remedy for many common ailments. Ginsenosides from ginseng have shown a wide range of biological effects, including antioxidant and anti-cancer properties. The goal of this project was to develop a method using electrospray ionization-mass spectrometry (ESI-MS) to identify and quantify seven ginsenosides in ginseng (*Panax quinquefolius* L., araliaceae). In addition, we sought to compare the quantities of ginsenosides in ginseng plant leaves and roots to determine in what part of the plant these compounds are more prevalent. Unlike other studies, our extraction methodology followed the typical extraction protocol most commonly utilized in the dietary supplement industry. This makes our findings relevant to the dietary supplements industry. The mass spectrometry method employed the negative ion and utilized an acetonitrile/aqueous acetic acid (1%) gradient. With this method, the ginsenosides were detected as negatively charged acetate clusters. Five leaf and their corresponding root extracts of cultivated, mature *Panax quinquefolius* plants were analyzed for ginsenoside content. It was determined that for ginsenosides Rb2 and Rd, there was a higher content in the leaves as opposed to the roots, while Rb1 was found to be more prevalent in the roots than in the leaves. A separate set of 1-year old ginseng seedling extracts were prepared by our collaborators and the same general trends were observed. These findings indicate that ginseng leaves are a viable source of ginsenosides,

which is significant given that native ginseng populations are threatened by overharvesting.

PART II:

Modern demands on agricultural production for food necessitate the use of pesticides such that without their use, upwards of $40 billion in crops would be lost. In the United States alone, the Environmental Protection Agency (EPA) reports an approximate use of 1.1 billion pounds of pesticides each year, approximately 20% of global pesticide usage. This creates a critical need for monitoring the presence of pesticides and their metabolites in soil effectively. Through collaboration with our colleagues at Syngenta Crop Protection, LLC, we have begun to develop a new method for this purpose. Our method is built off of the QuEChERS extraction methodology, and meets the demands for rapidness and cost effectiveness. Traditional methods employ very time consuming and costly sample preparation procedures that are unique to particular analytes. Our new method allows for the simultaneous detection and quantitation of 10 analytes of various chemical families, with the opportunity to expand further. Using ultra-performance liquid chromatography and mass spectrometry, we are able to detect a number of the pesticides of interest in 1 ppb spikes on soil.

ANALYTICAL DEVELOPMENT FOR ANALYSIS OF AMERICAN GINSENG AND MULTIPLE AGRICULTURAL CHEMICALS FROM SOIL

by

Michael B. Conine

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Approved by

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APPROVAL PAGE

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CHAPTER I

ANALYSIS OF GINSENOSIDES FROM ROOTS AND LEAVES OF AMERICAN GINSENG

Introduction

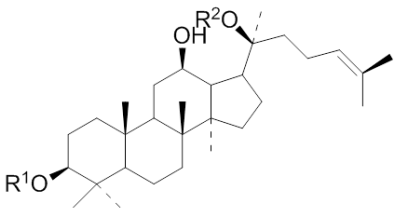
The ginseng plant has been utilized in traditional Chinese medicine for more than 2,000 years [1-9] with the belief that the plant is a panacea and promotes longevity and stamina [1, 6, 10-12]. In recent years, ginseng has been heavily used in dietary supplements, and in 2004 Luchtefeld *et al.* reported that ginseng accounted for about $300 million annually in the United States, or about 15-20% of the market share of dietary supplements [7]. It should be noted that a number of different species of ginseng are used medicinally, including *Panax ginseng* (Asian or Korean ginseng), *Panax quinquefolius* (American ginseng), *Panax notoginseng* (Tienchi or Sanchi ginseng), *Panax vietnamensis* (Vietnamese ginseng) and *Panax japonicas* (Japanese ginseng) [5, 13, 14]. Of these, *Panax ginseng* and *Panax quinquefolius* are the most commonly used [13, 14].

The studies described herein focus on American ginseng (*Panax quinquefolius*), which has grown in popularity and become one of the top species cultivated and has been employed for a variety of different purposes, such as anti-diabetes, anti-tumor, anti-aging

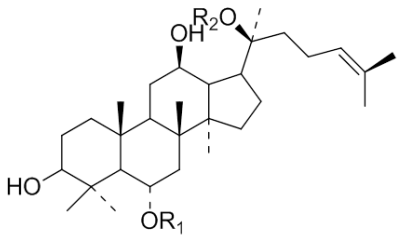
anti-stress, anti-cancer, and treatment of coronary heart disease, to name a few [6, 10, 13, 15-21]. American ginseng has been shown to possess an array of different chemical constituents. These include fatty acids, polysaccharides, peptides, polyactylenic alcoholsand ginsenosides [12, 14, 22, 23]. Of these, ginsenosides are considered to be the most important in dictating the biological activity of ginseng [4, 8, 12, 15, 19, 22-25]. These ginsenosides fall under the structural class known as triterpene saponins, which are comprised of triterpenoid aglycones with varying substituents (Figure 1.1 Structures of the eight ginsenosides being studied) [2, 23, 25-27]. Previous studies have identified over 30 different ginsenosides. This research is focused on the seven most abundant and commonly studied ginsenosides: Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 [2, 5-8, 10, 17, 19, 21, 22, 28-30].

A number of studies have demonstrated biological activity of various ginsenosides. For example, ginsenoside Rb1 was shown to possess phytoestrogen properties with breast cancer cells in vitro [6, 10, 13] as well as to have neuroprotective effects, and Rg1 has also shown similar central nervous system (CNS) effects [1, 13]. Ginsenoside Rg1 has also been shown to increase humoral and immune response in vitro [1]. Ginsenoside Re has been shown to exhibit antioxidant properties [2, 31] and ginsenoside Rg1 has shown immunomodulatory effects [32] in vitro, modulating cardiovascular function in vitro [33], as well as enhancing tissue regeneration both in vitro and in vivo [34], improving memory in vivo studies with rats [11] and potentially treating diabetes [22]. Finally, ginsenoside Rb2 has been shown under some conditions to reduce sterol regulatory element binding proteins and to stimulate RNA expression [27].

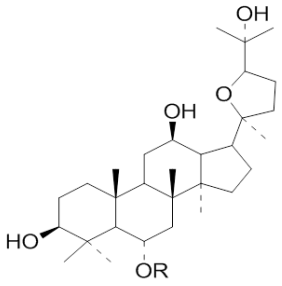
The effects mentioned here demonstrate only some of the diverse array of pharmacological effects attributed to ginsenosides, and ongoing research continues to identify and further characterize their other specific biological activities.



Ginsenoside	R^1	R^2	Formula
Rb1	-glc[2→1]glc	-glc[6→1]glc	$C_{54}H_{92}O_{23}$
Rb2	-glc[2→1]glc	-glc[6→1]ara(a)	$C_{53}H_{90}O_{22}$
Rc	-glc[2→1]glc	-glc[6→1]ara(f)	$C_{53}H_{90}O_{22}$
Rd	-glc[2→1]glc	-glc	$C_{48}H_{82}O_{18}$



Ginsenoside	R^1	R^2	Formula
Re	-glc[2→1]rha	-glc	$C_{48}H_{82}O_{18}$
Rf	-glc[2→1]glc	-H	$C_{42}H_{72}O_{14}$
Rg1	-glc	-glc	$C_{42}H_{72}O_{14}$



Ginsenoside	R	Formula
F11	-glc[2→1]rha	$C_{42}H_{72}O_{14}$

Figure 1.1. Structures of the eight ginsenosides being studied. All of the analytes are comprised of a common core structure, yet vary with their sugar substituents.

Over the past 30 years, there has been extensive research developing new methods for quantitation and identification of ginsenosides towards the goal of developing accurate methods for quality control of ginseng products [14]. Traditionally,

gas chromatography coupled with mass spectrometry (GC-MS) methods were utilized, but due to the extensive derivatization steps that are needed for analysis and low signal response of target ions, high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) has become the method of choice [3, 14, 15]. With this study, we applied quantitative HPLC-ESI-MS to determine the quantities of seven ginsenosides in American ginseng leaf and root extracts of *P. quinquefolius* plants cultivated in several different environments and of varying age. Given that ginseng is threatened and overharvested in much of its native habitat [35], our goal was to investigate whether the leaves of cultivated ginseng (*P. quinquefolius*), which can be harvested in a sustainable fashion (without killing the plants), could serve as a viable source of ginsenosides. Previous studies have compared ginsenoside levels in different *P. quinquefolius* plant parts, or in plants of different ages [9, 12, 19]. However, none of these studies have employed extraction methods consistent with those used in the dietary supplements industry. Our goal was to fill this gap in the literature, demonstrating differences in ginsenoside levels in extracts prepared from roots and leaves of various individual ginseng plants with procedures consistent with dietary supplements manufacture.

Experimental

Chemicals and Reagents

Standards for seven ginsenosides were obtained from ChromaDex (Irvine, CA, USA, purity >89%): Rb1, Rb2, Rc, Rd, Re, Rf and Rg1. Glacial Acetic Acid (≥99.7% purity) and ACS/USP grade ethanol were obtained from Fisher Scientific (Hampton, NH, USA). Water was purified with a Barnstead Nanopure Diamond filter system (Thermo, San Jose, CA, USA). UV/HPLC grade acetonitrile was obtained from Pharmco-Aaper (Brookfield, CT; Shelbyville, KY, USA).

Preparation of Ginseng Extracts

Individual ginseng plants (5) were obtained from Bearwallow Ginseng (Hendersonville, NC). A voucher specimen is retained at the University of North Carolina Herbarium (NCU602024). The plants were dried at room temperature and the leaves and roots were ground separately using an IKA (IKA, Wilmington, NC, USA) M20 Universal Mill grinder. Extracts were prepared in 50:50 ethanol to water consistent with standard practices in the dietary supplements industry[36] at 1:10 (w/v) ratio of plant material to solvent. Samples were macerated for 45 hrs, vacuum-filtered to remove solid plant material, and air dried to constant mass. Stock solutions of 1 mg/mL ethanol were prepared for each extract for analysis via HPLC-MS. Dried plant extract materials were placed in glass vials and stored at room temperature in the dark.

A set of 15 one-year old ginseng seedlings were prepared by our collaborators at Salem State. Leaf and root extracts were prepared separately in which forty milligrams of

plant were crushed and sonicated in three milliliters of absolute ethanol. After sonication, the extract samples were filtered and stored in plastic vials at 5 ºC prior to analysis.

Analysis via HPLC/ESI-MS

Samples of Ginseng were analyzed with an HP1100 HPLC (Agilent, Santa Clara, CA, USA) at a flow rate of 0.2 mL/min for a total run time of 40 min. A reverse phase Alltech (Grace, Deerfield, IL, USA) Prevail C18 column (50 x 2.1 mm i.d., 3µm) was used for separation. The mobile phase consisted of an acetonitrile and 1% acetic acid nanopure water gradient with the following composition: 0-3 min 95% water, 3-7 min 95-75% water, 7-30 min 75-60% water, 30-35 min 60-5% water, 35-35.1 min 5-95% water, and 35.1-40 min 95% water. The HPLC was coupled online to an LCQ Advantage Ion Trap (Thermo, San Jose, CA, USA) mass spectrometer with electrospray ionization source. Detection was performed utilizing negative ion mode with a scan range of 50 – 2000 m/z. Instrument parameters are as follows: capillary temperature 275ºC, sheath gas flow of 20 arb, source voltage of 4.50 kV, capillary voltage of -10.00 V, and tube lens offset of -50.00 V.

Identification of Ginsenosides

A standard mixture of all ginsenoside standards was analyzed to obtain the characteristic retention time of each standard. After optimal separation was accomplished, extract samples were analyzed and compounds were identified by matching retention times and m/z values to those of the standard mixture.

Quantification of Ginsenosides

External calibration curves were plotted from the seven ginsenoside standards from a concentration range of 0.12 µM to 15.6 µM, above which concentration signal saturation was observed. Calibration curves were plotted as average peak area for the relevant selected ion trace (for duplicate injections) versus concentration. Extract samples were prepared at several dilutions (25, 50, 100 and 200-fold) and analyzed in duplicate injections with the same method employed for the standards. Dilutions that fell within the linear range of the calibration curve were used for quantitation.

Results and Discussion

Chromatographic Method Optimization

As seen in Figure 1.2 Chromatograms of standard mixture, leaf sample and root sample, all ginsenoside standards could be separated chromatographically with the method employed except Rg1 (859.50 m/z) and Re (1005.56 m/z). Ginsenosides were detected in the negative ion mode as both the deprotonated molecular ion $[M-H]^-$ and the acetate cluster $[M+CH_3COO]^-$.

Differences in the masses of these ions for Rg1 and Re enabled them to be distinguished and separately quantified despite co-elution as seen in Figure 1.3 Chromatogram of co-eluting peaks Re and Rg1. The prevalence of acetate clusters was due to the addition of acetic acid in the mobile phase to optimize separation, and comparison of the mass of the $[M-H]^-$ ion and $[M+CH_3COO]^-$ ion provided additional information for verifying identity of the ginsenosides detected.

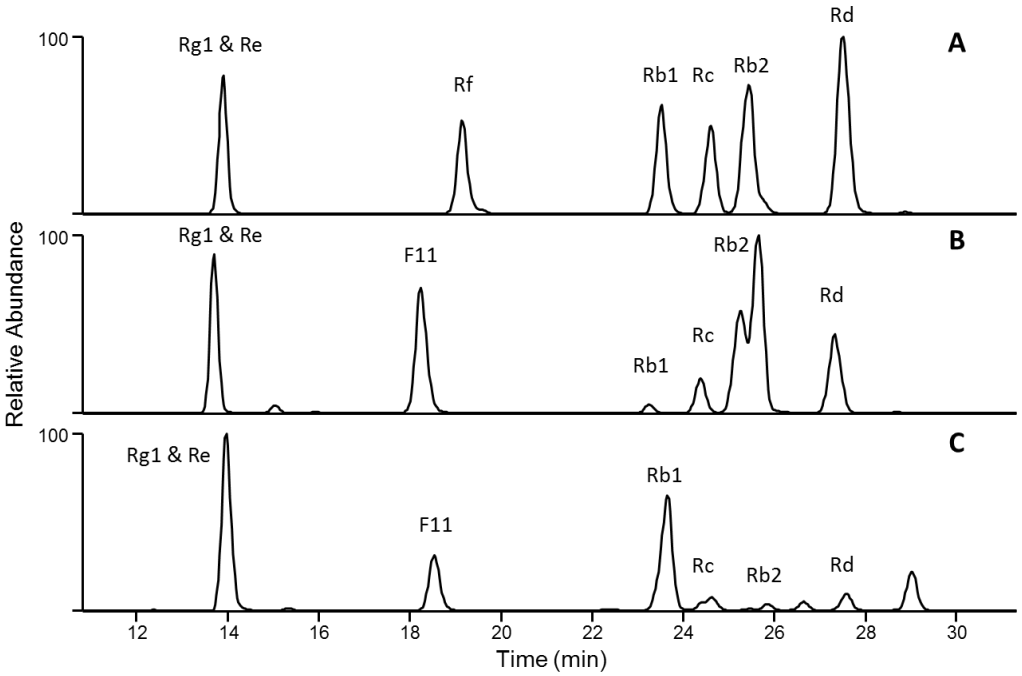


Figure 1.2. Chromatograms of standard mixture, leaf sample and root sample. A) Standard mixture of all seven ginsenoside standards from Chromadex B) Leaf extract sample from mature ginseng plant C) Root extract sample from mature ginseng plant

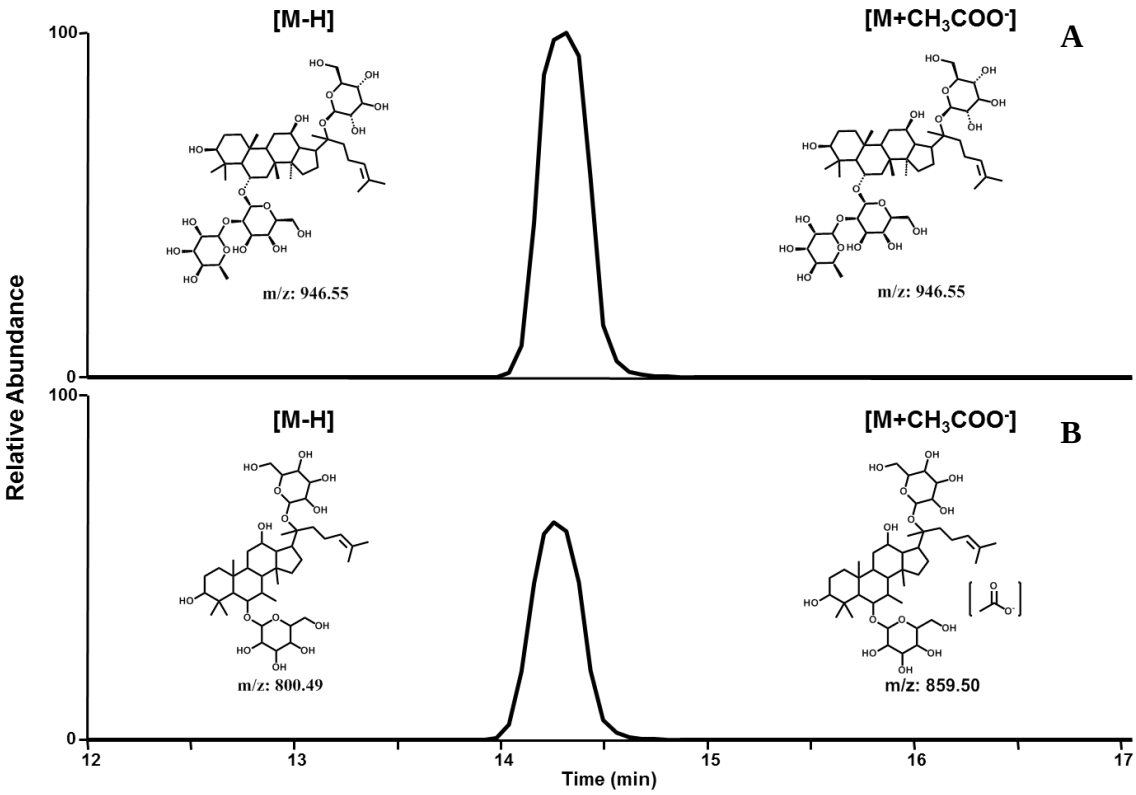


Figure 1.3. Chromatogram of co-eluting peaks Re and Rg. Ginsensoside Re is seen in chromatogram A and ginsenoside Rg is seen in chromatogram B. Analyte structure shown for deprotonated molecular ion, as well as the acetate cluster ion with an additional 59 m/z.

All six of the ginsenosides for which standards were available (Figure 1) could be detected in both leaf and root samples with one exception. There was a seventh peak in the extract samples that eluted at 18 min. It was found that this peak in the extract samples actually corresponds to a separate ginsenoside known as F11, as documented in the literature [7, 13, 15, 19, 20]. F11 has been commonly mistaken as a false positive for Rf.

Relative Ginsenoside Content in Leaves and Roots

Figure 1.4 Relative weight percent of all six ginsenosides in root and leaf extracts from mature ginseng plants, shows the distribution of the different ginsenosides in the leaves and roots for the mature *P. quinquefolius* plant extracts. From this graph it can be seen that for ginsenosides Rb2 and Rd, the presence of these ginsenosides were significantly more in the leaves than the roots based on a student's T-test analysis, indicating that the leaves can be used as a viable source for these two ginsenosides. Alternatively, ginsenoside Rb1 is more abundant in the roots than the leaves, indicating that the roots may still be the best source of this compound.

A separate set of extracts were analyzed containing one-year old seedlings from a different location within North Carolina as prepared by our collaborators as seen in Figure 1.5 Relative weight percent for all six detectable ginsenosides from a separate set of fifteen 1-year old ginseng seedlings. A general trend was seen that was similar to our results aforementioned, where some of the ginsenosides were more abundant in the leaves than in the roots. However, no firm conclusion or comparison can be made due to the variability with age of plants, genetic material, and extraction protocols among other variables. It was encouraging to see that even with the change in these conditions, the relative amounts of these precious ginsenosides was seen.

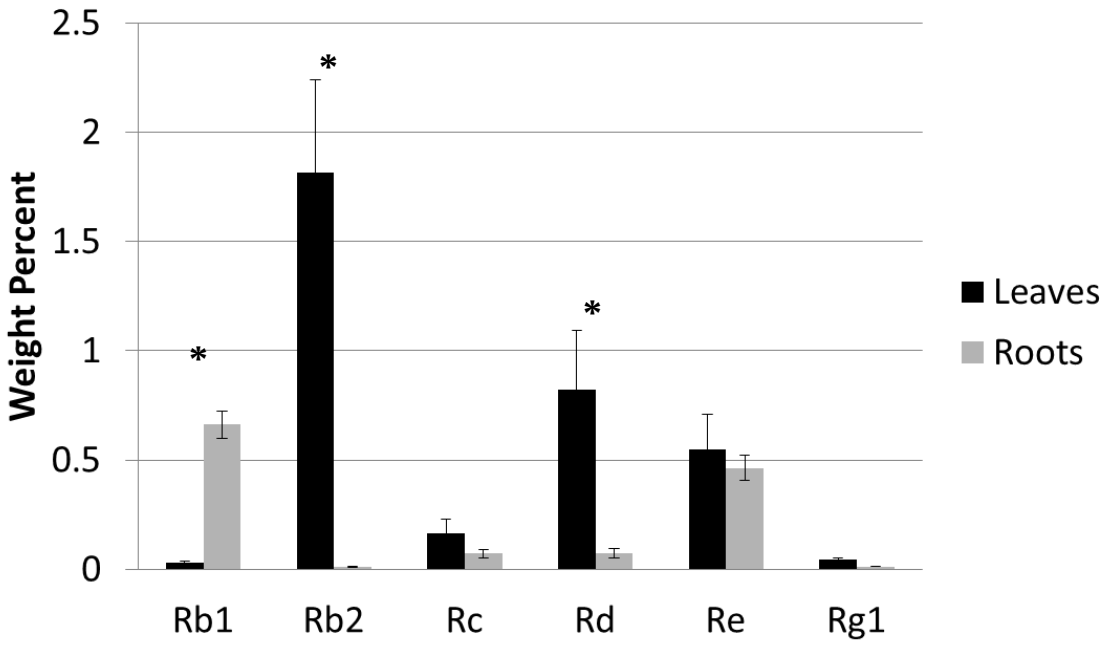


Figure 1.4. Relative weight percent of all six ginsenosides in root and leaf extracts from mature ginseng plants. Data shows results from 5 individual *Panax quinquefolius* root and leaf samples of North Carolina cultivated ginseng. * indicates a statistical difference between leaf and root samples verified by the use of a Student's T-test.

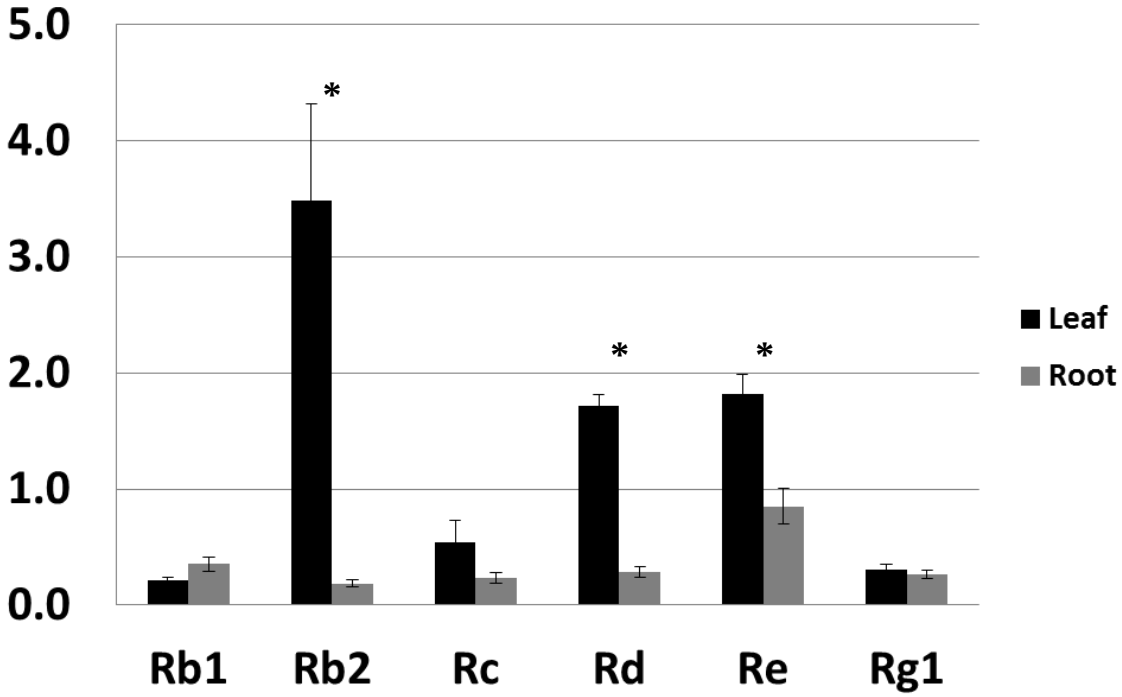


Figure 1.5. Relative weight percent for all six detectable ginsenosides from a separate set of fifteen 1-year old ginseng seedlings. Extracts were prepared differently than the mature plant extracts seen in Figure 1.4 Relative weight percent of all six ginsenosides in root and leaf extracts from mature ginseng plants. * indicates a statistical difference between leaf and root samples verified by the use of a Student's T-test.

The roots of ginseng plants have traditionally been the main source for ginsenosides and these findings indicate that ginseng leaves may be a viable source for these particular ginsenosides, which is significant given the fact that native ginseng populations are threatened by overharvesting.

Conclusion

Summary and Significance

This research project focused on developing an analytical technique that would allow for the separation, detection and quantitation of different active compounds in American ginseng (*Panax quinquefolius*) root and leaves known as ginsenosides. Seven ginsenosides were originally targeted for quantitation, Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1. An HPLC-MS method was made using a C18 stationary phase and mobile phase gradient composed of acetonitrile and 1% acetic acid aqueous solution. All of the major ginsenosides except Rf (which is known to be produced by *Panax ginseng* but not *Panax quinquefolius*) were detected in the *P. quinquefolius* samples. In addition, ginsenoside F11 (for which there is no available standard) was determined to be present in the samples, consistent with literature reports.

A comparison study was completed of five separate North Carolina mature ginseng plant roots with their corresponding leaves. It was seen that for ginsenosides Rb2 and Rd, there was a significantly greater amount of these ginsenosides present in the leaves compared to the roots, whereas ginsenoside Rb1 was significantly more abundant in the roots than the leaves. Similarly, a separate set of 1-year old ginseng seedlings were prepared by our collaborators at Salem College. These extracts followed a different extraction procedure, yet the relative amounts of these seven ginsenosides followed the same trend that we saw in our extracts, which was encouraging to see that a change in extraction techniques showed similar results. All of our results were significant in regards to the leaves of ginseng plants serving as a possible resource for the harvesting of

ginsenosides from different ginseng plants, as native ginseng populations are threatened by overharvesting.

CHAPTER II

SIMULTANEOUS ANALYSIS OF MULTIPLE AGRICULTURAL CHEMICALS FROM SOIL

Introduction

Modern demands on agricultural production for food necessitate the use of agro chemicals including pesticides and herbicides, such that without their use, crop production could decrease up to 40% [37]. In the United States alone, the Environmental Protection Agency (EPA) reports an approximate use of 1.1 billion pounds of these agro chemicals each year, approximately 20% of global usage. This creates a critical need for monitoring the presence of agro chemicals and their metabolites in soil effectively. Repetitive exposure of soil to these chemicals can lead them to build up in the environment. Some herbicides, such as prodiamine, have little to no toxicity towards humans, while other compounds pose greater hazard levels to humans, for example, the herbicide diquat is fatal when ingested. The wide array of effects agro chemicals can exhibit on their surroundings drives the need for a rugged extraction method that will allow for multiple classes to be analyzed in a single trial. An ideal method will analyze multiple compounds simultaneously, but at the same time have a rapid turnaround and be cost effective and simple to use.

Environmental Monitoring of Agro Chemicals

The agro chemical industry is enormous, with annual sales of approximately $40 billion [38]. This massive yearly usage spans over 500 different active ingredients [39]. It is easy to understand why agro chemicals play a vital role in agriculture – without their use production demands for food would not be met [40]. However, a portion of the chemicals applied to agricultural crops end up in the surrounding environment, including the air, soil, and groundwater. Careful monitoring is needed to ensure that these chemical residues do not cause adverse effects on wildlife and humans [39, 41-43]. To accomplish this monitoring effectively, there is a great need for methods to measure the levels of these agro chemicals on crops and in the surrounding environment.

The most common methods that have been employed since the 1960's are known as multi-residue extraction methods, and seek to analyze multiple compounds [40, 44, 45]. Such methods exist for simultaneous quantification of different agro chemicals across a wide array of matrices ranging from corn to meat [40, 46-51]. There are, however, very few multi-residue methods that are adapted for use with soil, due in large part to its matrix complexity [41-43, 46, 50, 52-56]. Techniques that have traditionally been used for soil analysis include the most common shaker method, Soxhlet extraction, ultrasonic solvent extraction (USE), pressurized liquid extraction (PLE), shake-flask extraction, microwave assisted extraction (MAE) and superficial fluid extraction (SFE), among others [43, 46, 52, 53, 56, 57].

Current protocols employed by our collaborators at Syngenta are very time consuming. With some methods, one technician can analyze only 15 samples in a single

day, due in large part to the need for extensive sample preparation. There are roughly 16 different steps that are needed for sample extraction before analysis by either gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS). In addition to being very time consuming and labor intensive, most existing methods for soil analysis employ a large quantity of organic solvents, such as acetone, methylene chloride, ethyl acetate, methanol, and dichloromethane. This ultimately creates vast quantities of waste, which is not ideal for the environment and is also very expensive [43, 45, 46, 53-57].

In 2003, Anastassiades and Lehotay developed a technique that was quick, easy, cheap, effective, rugged and simple (QuEChERS), which addressed these issues [44]. Originally designed for the determination of the various types of agro chemicals in produce, the QuEChERS methodology has been extensively applied and validated for analysis of different agro chemicals in many other media [45, 47-51, 58]. The goal of this research project is to develop a method that allows for simultaneous analysis of diverse compounds across several soil types, something currently lacking in the field.

Research Objectives

There are two main objectives of this research project. The first objective is the development of a robust ultra-performance liquid chromatography mass spectrometry method (UPLC/MS/MS) that ideally will allow for the simultaneous quantification of 14 different pesticide analytes of interest (Figure 2.1 Structures of the 14 analytes of interest) that are all classified as herbicides. The importance of these analytes is two-fold. The first reason is that all of these target compounds are active ingredients produced by our

collaborators at Syngenta Crop Protection, LLC and are the most frequently requested products for soil analysis by Syngenta customers. The second reason is that these 14 analytes vary in their chemical composition and structural class. With the development of this new method, a major goal is to quantify multiple analytes of these diverse structural classes simultaneously.

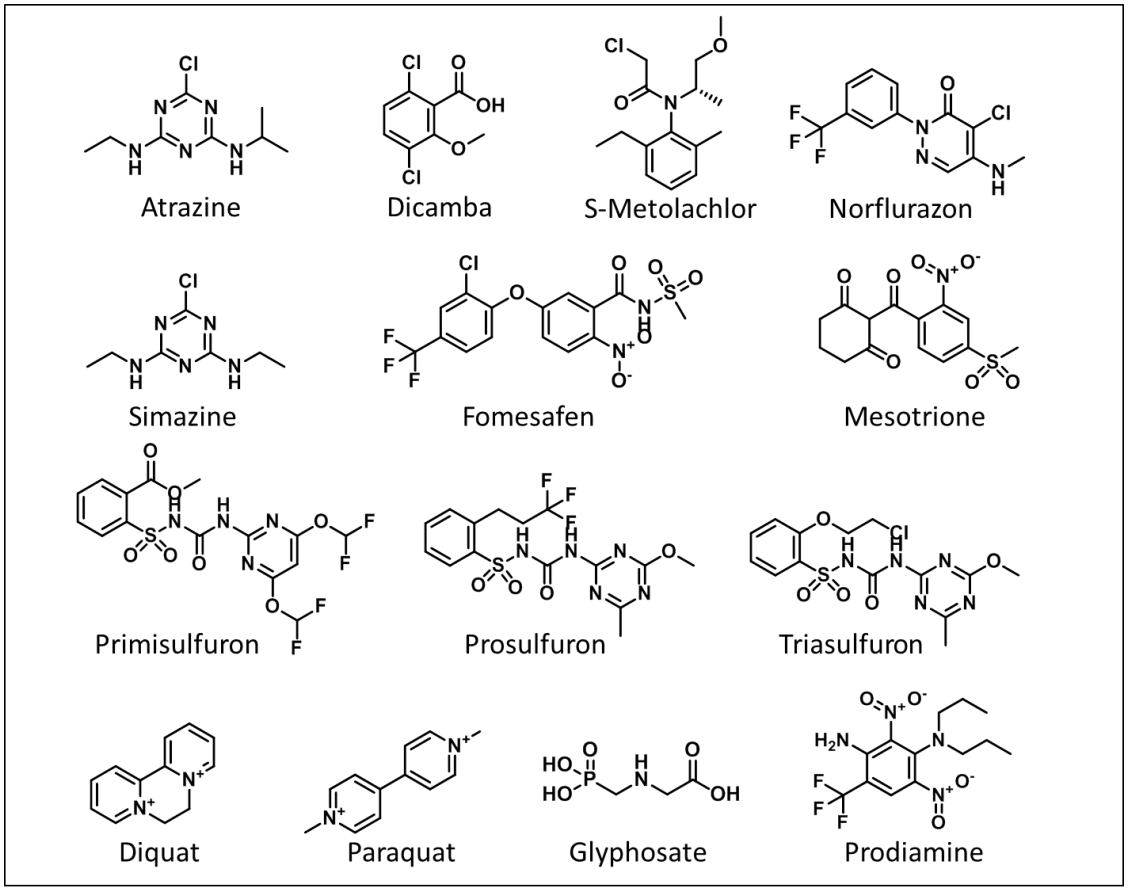


Figure 2.1. Structures of the 14 analytes of interest. Compounds here represent 14 active ingredients produced by Syngenta Crop Protection, LLC.

Our desired outcomes for the extraction method include greater than seventy percent recovery of these agro chemicals from soil, a recovery standard deviation less than twenty percent and to obtain no more than thirty percent matrix suppression.

Multi-Residue Methods for Agro Chemical Analysis

As previously stated, multi-residue methods for analyzing agricultural chemicals have been used for over 50 years. The first of its kind was the Mills method that began use in the 1960's [40, 44, 45]. The Mills method and the other initial methodologies used were developed to target many organochlorine, organophosphorous, and organonitrogone insecticides, but quickly progressed to widen the analytical capabilities to include a much more diverse polarity range [44, 45]. In the 1980's, there was a major shift from the original procedures to those that would reduce the use of chlorinated solvents, due to environmental and health concerns [44, 45]. This trend continued to grow through the 1990's and it has led to the development of new techniques as mentioned previously. The development of QuEChERS has had a tremendous impact for sample preparation owing to its many advantages including high recoveries (>85%), accurate and precise results, high-throughput (10-20 samples in about 30 minutes), small solvent and labware waste, simple and easy method, rugged methodology, and cheap (only about $1 of materials used for 10g of sample) [44-46, 59]. Although QuEChERS was originally developed for use on fruits and other plants, there has been some application of this method on soils. However, soil analysis using QuEChERS methodology is very limited [41-43, 50, 52-56]. Of the reported instances of QuEChERS analysis with soil, they have been applied to individual case studies of different agro chemicals. None of these instances have been

utilized for simultaneous identification and quantification of multiple pesticide classes [41-43, 50, 52-56]. This project was designed in order to fill the need for a multi-residue method that allows for simultaneous extraction of diverse agro chemicals in soil.

Overview of Techniques Used in this Research

As previously mentioned, there are two main objectives of this research project. First we seek to develop and implement a robust UPLC/MS/MS method that will allow for the identification and quantification of multiple target agro chemicals. For the most effective and sensitive identification and quantitation, we will utilize selected reaction monitoring (SRM) mass spectrometry [60]. SRM allows for greater selectivity and sensitivity than full scan mass spectrometry by specifically targeting a desrired mass of a particular analyte in a triple quadrupole mass spectrometer. Target precursor ions (typically the [M+H]+ or [M-H]- ion for the analyte of interest) are selected by the first quadrupole mass analyzer. These ions are fragmented into product ions in the second quadrupole, and the specific target product ions are separated out from all other product ions in the third quadrupole, enabling their selective detection [61]. To be consistent with industry standards and application for our collaborators, we used the heated electrospray ionization (HESI-II) probe. The use of this probe allows for faster desolvation of the solvent in our sample, providing a more rapid analysis.

Ultra performance liquid chromatography (UPLC) was employed to accomplish the separations necessary for these experiments. The main advantage of using UPLC as opposed to traditional gas chromatography (GC) is that UPLC eliminates much of the sample preparation needed for analysis, in addition to being much more sensitive and

having a higher selectivity for analysis of complex samples [52, 57]. UPLC was also chosen over the standard high performance liquid chromatography (HPLC) in large part to the increase in resolution that allows for shorter run times. UPLC utilizes smaller pore size in the column, which ultimately increases the plate number within the column, allowing for more interaction between the analytes and the stationary phase. Due to the smaller pore size, there is also a great increase in pressure within the column, which has only been made feasible in recent years, allowing this technology to be used more frequently in the industry [61]. Typically UPLC utilizes a C18 stationary phase column [52, 53], which we incorporated with our method development in order to maintain a close similarity to the methods of our collaborators at Syngenta Crop Protection, LLC.

Experimental

Preparation and Storage of Standards

Our collaborators at Syngenta Crop Protection LLC provided us with solid standards of each of the 14 analytes of interest (>95% purity). Stock solutions were prepared from these dry standards at concentrations of 1 milligram of solid standard in 1 milliliter of acetonitrile. Simazine was prepared using methanol as solvent instead of acetonitrile due to solubility issues. Serial dilutions were prepared from these stock standards using a ratio of 90:10, water to acetonitrile solution. Optima grade LC/MS water, acetonitrile and methanol were obtained from Fisher Scientific. All solutions were stored in a -20ºC freezer. Standards for HILIC development including paraquat, diquat

and glyphosate, were comprised of a 90:10, acetonitrile to water solution, at a starting concentration of 1ppm and serial dilutions were performed from these stock solutions.

Extraction Protocol

Our collaborators at Syngenta Crop Protection LLC prepared three different soil sets there were used throughout this research. The clay loam soil set was obtained from Marysville, Ohio. The silt loam soil was obtained from Sarpy, Nebraska. The sandy loam soil was obtained from Conklin, Michigan. Upon receiving samples, each soil type was dried down in triplicate in order to determine the moisture content of each. It was determined that each soil type inherently contained 12% moisture content. For further experiments each soil that was analyzed was adjusted to an overall start of 30% moisture based on the literature [42, 43, 62].

Materials for the QuEChERS based extraction methodology were obtained from Agilent Technologies (Santa Clara, CA). Extraction sorbents consisted of 4 grams magnesium sulfate (purity >98.5%) and 1 gram sodium chloride (purity >99.5%). There were two dispersive-SPE cleanup sorbents that were analyzed. The first was a mixture of 150 milligrams magnesium sulfate (purity >98.5%) and 50 milligrams of a primary-secondary amine (PSA). The second sorbent that was analyzed consisted of 150 milligrams of magnesium sulfate and 25 milligrams of C18.

Initially a 10g sample of soil is measured, prepared to an overall 30% moisture content and a spike of desired analyte concentration is put on the soil. After sitting for 30 minutes, the extraction procedure begins with the addition of 10 mL of acetonitrile and the sample is shaken for one minute. Next, 4g of magnesium sulfate and 1g of sodium

chloride are added to the mixture in order to create a liquid-liquid partition between any aqueous solvent present and the acetonitrile solvent that was added. This step is meant to push the analytes of interest into the organic acetonitrile layer. After the salts are added the entire mixture is centrifuged for 5 minutes at 5000 rpm. From here, the sample undergoes a dispersive-SPE cleanup step in order to minimize any background matrix effects, and then the sample is analyzed via the UPCL-MS/MS method previously developed.

Extract Analysis via UPLC-MS/MS

All standards and extraction samples were analyzed using reverse phase UPL-MS/MS. A Waters Acquity Ultra Performance LC system was used for the chromatography analysis (Milford, MA). An Acquity UPLC BEH shield RP18 1.7 µm 2.1 x 50 mm column was employed for the separation of samples (Milford, MA). The mobile phase consisted of a gradient method using Optima grade acetonitrile with 0.1% formic acid and Optima grade water with 0.1% formic acid to facilitate ionization. The gradient composition is as follows: 0-1.00 min 90% water, 1-3.5 min 90-50% water, 3.5-8 min 50-20% water, 8-9 min 20-90% water, 9-10 min 90% water. A flow rate of 0.3 mL/min was used for a total run time of 10 min.

A Thermo Scientific Heated Electrospray Ionization (HESI-II) probe (Thermo, San Jose, CA) was utilized as the ionization source. The source was operated with a vaporizer temperature of 250ºC, which allowed for a more efficient desolvation process. The ionization source was coupled a Thermo Scientific TSQ Quantum Access Triple Quadrupole mass spectrometer (Thermo, San Jose, CA) operating in selected reaction

monitoring mode. Utilizing this unique feature of the triple quadrupole mass spectrometer allows for a sensitivity boost in signal for analytes of interest by specifically scanning for certain ions, as previously described and shown in Table 2.1 Chart of all target analytes with their corresponding precursor ions, confirmatory product ions, collision energies and retention times.

Results and Discussion

Mass Spectrometry Method Development

It was initially necessary to optimize the mass spectrometry conditions that would allow for all desired analytes to be detected. Each analyte was initially analyzed individually in order to identify the instrument parameters that allowed for the accurate identification of each precursor ion for all 14 analytes (Figure 2.1 Structures of the 14 analytes of interest). After identification of each precursor ion, these ions were fragmented into a set of product ions in order to identify two confirmatory ions for each analyte. The product ions that were selected met two criteria: the ion signal had to be strong enough to be detected and the ion had to be unique to the precursor ion. Table 2.1 Chart of all target analytes with their corresponding precursor ions, confirmatory product ions, collision energies and retention times, shows each analyte with their precursor ion and subsequent product confirmatory ions that was used to build our UPLC-MS/MS method.

As previously stated, each analyte was analyzed individually to determine an appropriate precursor ion and confirmatory product ions and to measure retention time.

To determine the product ions, each analyte was directly injected into the mass spectrometer and ionization conditions were optimized, allowing different product ions to form. After the precursor ion was fragmented a graph like that in Figure 2.2 Product ion breakdown of triasulfuron, 402.1 m/z, was produced.

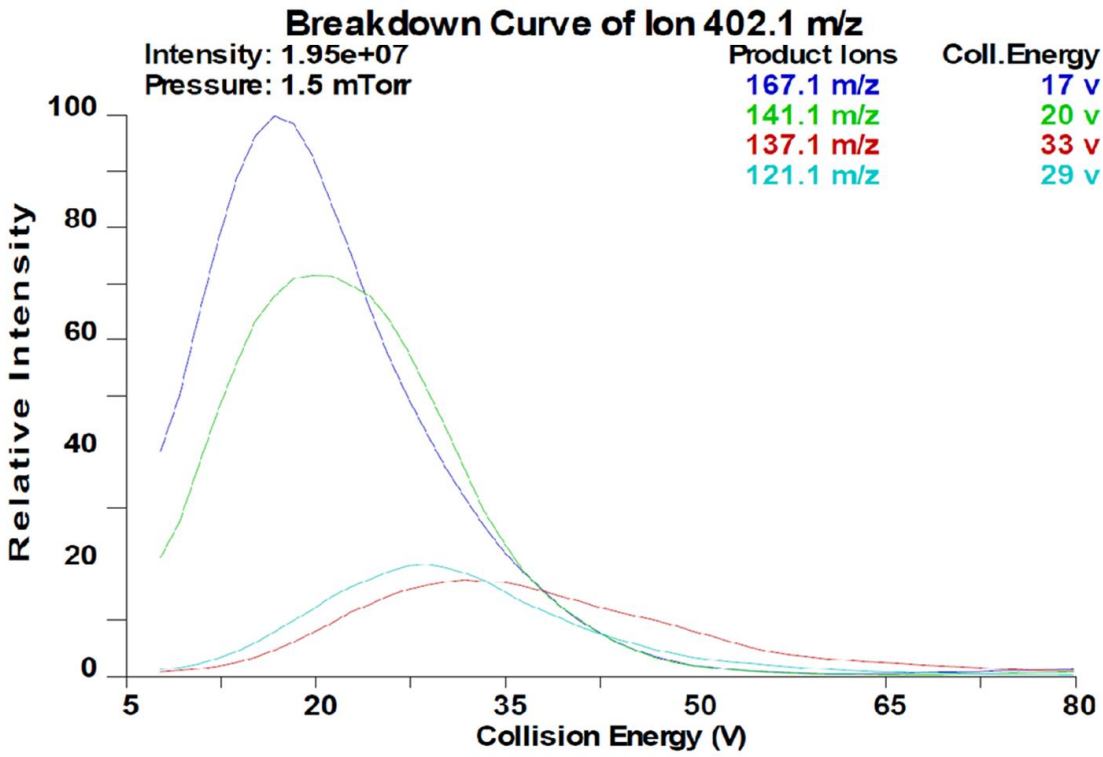


Figure 2.2. Product ion breakdown of triasulfuron. This graph is representative of the process used in determining appropriate product ions to monitor in SRM experiments for each of the 14 analytes of interest.

From here the most unique product ions were determined for each analyte. These ions selected serve as the confirmatory ions for each analyte of interest and were unique

to each specific precursor ion in order for no overlap during analysis of a complex mixture of analytes. All information regarding precursor and product ions can be found in Table 2.1. The data from this table was used to create the mass spectrometer SRM method.

Table 2.1. Chart of all target analytes with their corresponding precursor ions, confirmatory product ions, collision energies and retention times

Pesticide	Mode	Precursor Ion (m/z)	Product Ion 1 (m/z)	Collision Energy (eV)	Product Ion 2 (m/z)	Collision Energy (eV)
Atrazine	Pos	216.1	174.2	17	104.2	27
Dicamba	Neg	219	175.1	7	144.9	13
Diquat	Pos	183.1	157.2	21	130.3	31
Fomesafen	Neg	437.1	194.9	40	221.9	33
Glyphosate	Neg	168				
Mesotrione	Neg	337.9	291.1	16	212	32
Norflurazon	Pos	304.1	284.1	23	160.1	30
Paraquat	Pos	186.1	171.3	19	155.2	37
Primisulfuron	Pos	469.0	199.2	23	254.1	17
Prodiamine	Neg	349.2	231.7	24	185.2	11
Prosulfuron	Pos	420.1	141.1	18	167.3	16
Simazine	Pos	202.1	132.1	18	124.2	18
S-Metolachlor	Pos	284.2	252.1	15	176.2	26
Triasulfuron	Pos	402.1	167.1	17	141.1	20

Liquid Chromatography Method Development

As stated previously, the goal of this research project was to develop a single method that would allow for the simultaneous analysis of 14 different analytes of interest. Initial experiments investigated the feasibility of determining all 14 standards in a single complex mixture using a traditional reverse phase C18 stationary phase with a mobile phase gradient as described in the *Methods* section. After a significant amount of effort towards method validation, it became evident that this method would be effective only for a subset of the analytes. Of the 14 analytes, ten could be detected (at varying limits of detection). These were: atrazine, simazine, dicamba, primisulfuron, prosulfuron, triasulfuron, S-metolachlor, mesotrione, norflurazon, and fomesafen. These analytes could all be detected and separated using UPLC coupled to a triple quadrupole mass spectrometer. Figure 2.3 Chromatogram of separated positive mode analytes, shows the chromatographic separation of the seven analytes from among these that could be detected in the positive ion mode, while Figure 2.4 Chromatogram of separated negative mode analytes, shows the ones that could be detected in the negative ion mode.

Reverse phase chromatography consists of a non-polar stationary phase and a polar mobile phase that gradually shifts throughout the separation to become less polar. This allows molecules that contain non-polar characteristics to adhere to the column, causing them to be retained. As the mobile phase becomes less polar the molecules that were adsorbed to the column are displaced due to interactions of the solvent with the stationary phase. Problems arise with this process when the analyte becomes more polar, preventing it from being retained on the stationary phase. Three of the analytes that could

not be easily determined with the C18 stationary phase, paraquat, diquat and glyphosate, all present this aforementioned difficulty. When looking at these structures (Figure 1), it can be clearly seen that they are much more polar than the rest of the analytes due to the fact that they contain inherent charges or more complex structures that include hydroxyl groups and phosphorus groups.

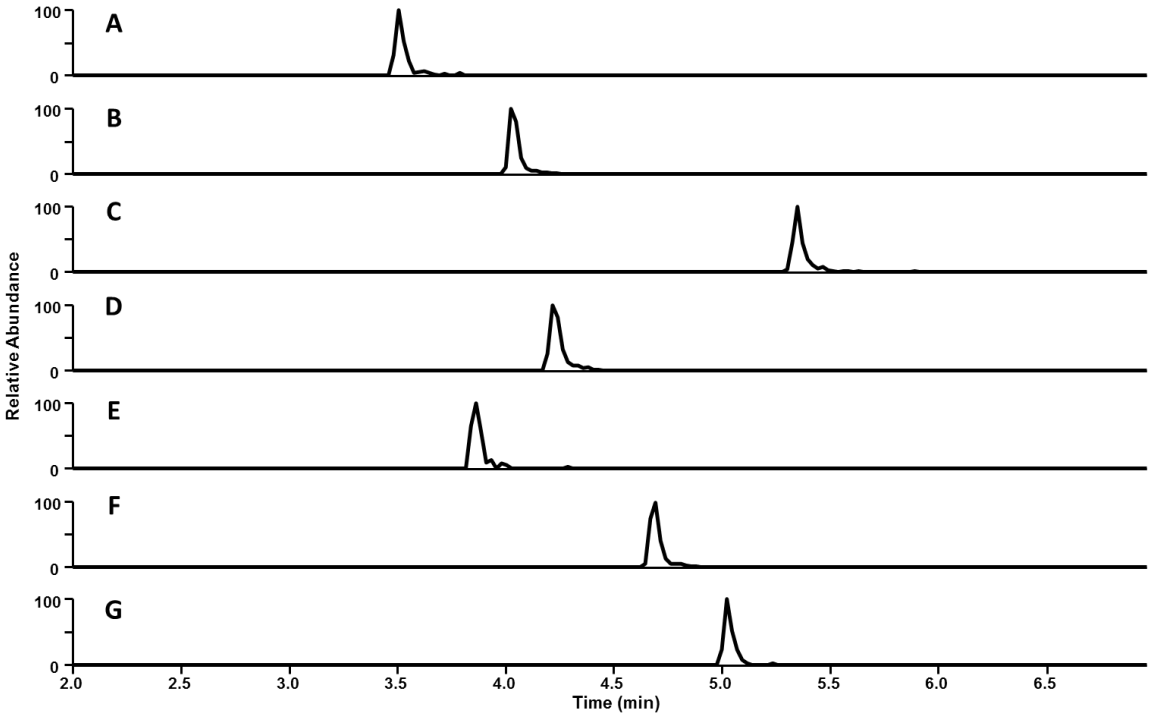


Figure 2.3. Chromatogram of separated positive mode analytes. Chromatograms show the seven target analytes that are detected in the positive ion mode from a 10 ppb stock solution. **A)** Simazine, 202.1 m/z **B)** Atrazine, 216.1 m/z **C)** S-metolachlor, 284.2 m/z **D)** Norflurazon, 204.1 m/z **E)** Triasulfuron, 402.1 m/z **F)** Prosulfuron, 420.1 m/z **G)** Primisulfuron, 469.0 m/z.

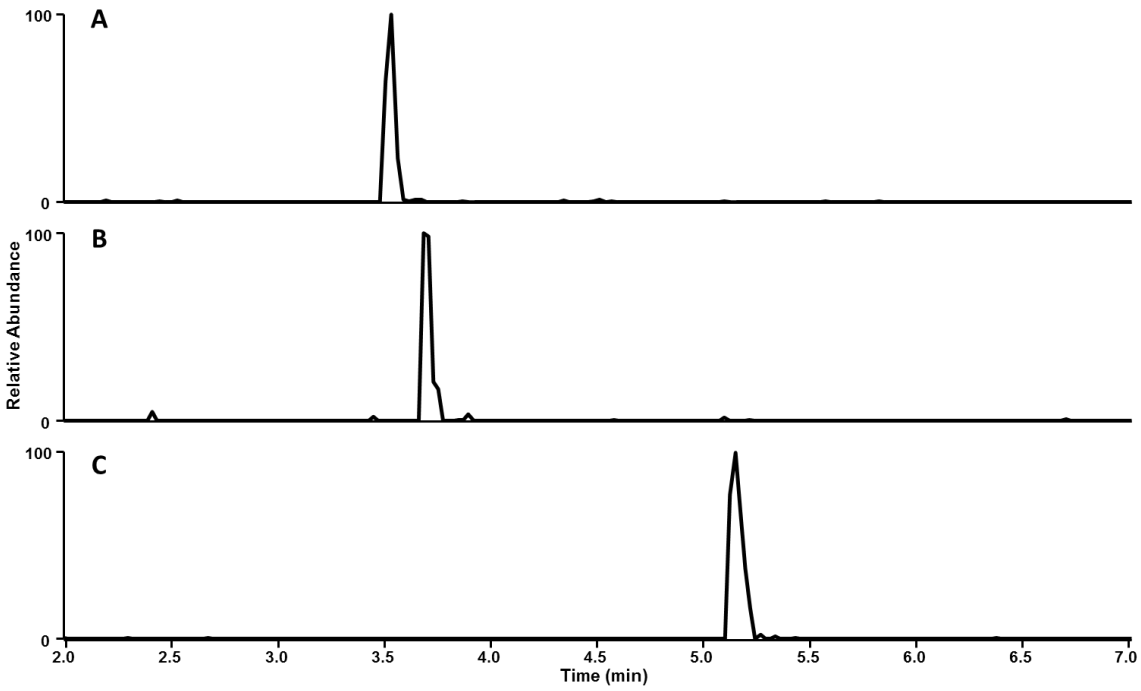


Figure 2.4. Chromatogram of separated negative mode analytes. Chromatograms show the three target analytes that are detected in the negative ion mode from a 10 ppb stock solution. **A)** Dicamba, 219.1 m/z **B)** Mesotrione, 337.9 m/z **C)** Fomesafen, 437.1 m/z.

To address the issue of poor retention of the polar analytes, it will be necessary to develop a second chromatographic method that specifically targets more polar compounds, such as these. We have begun developing such a method that utilizes a newer chromatographic separation technique known as hydrophilic interaction liquid chromatography (HILIC). HILIC utilizes a strongly polar stationary phase that is activated by an aqueous layer from the mobile phase. This activation allows for a liquid-liquid like partitioning to occur that facilitates the retention of these strongly polar analytes [61, 63]. HILIC is advantageous for this application because polar organic molecules, like our analytes of interest, are retained more effectively than they are in a C18 stationary phase. Initial tests have shown positive results for retaining paraquat with a HILIC stationary phase, as seen in Figure 2.5 Chromatogram of paraquat utilizing HILIC chromatography. Continuing efforts are being focused on further developing a more complete method that will allow for the retention and separation of diquat and glyphosate.

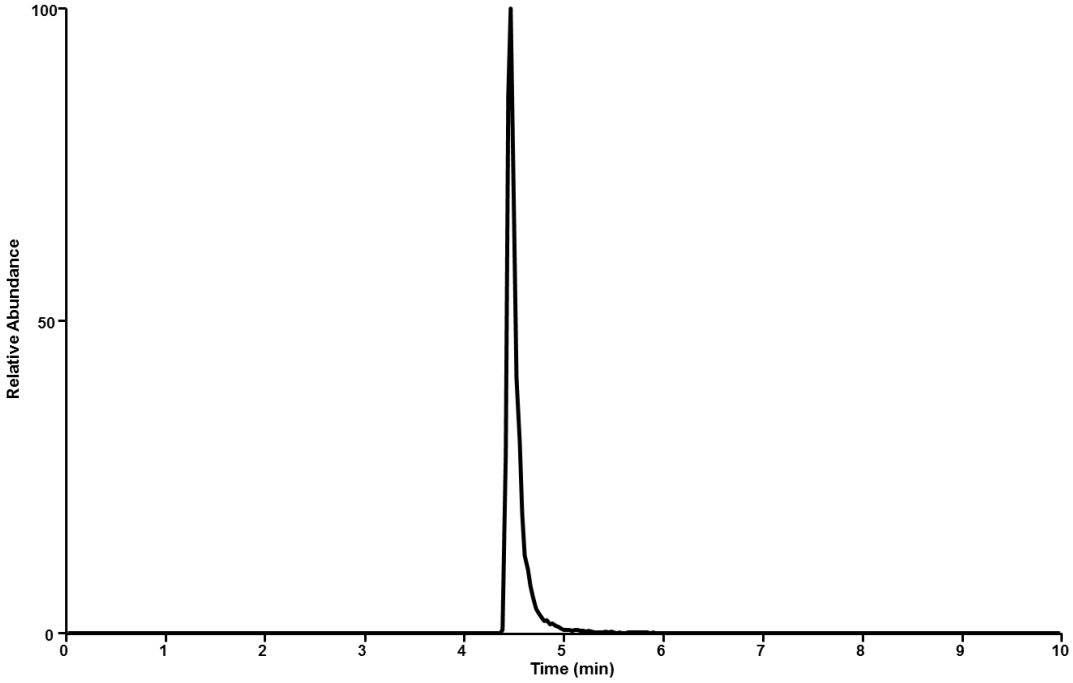


Figure 2.5. Chromatogram of paraquat utilizing HILIC chromatography. Chromatogram shows the initial successful retention results for retention of a 1 ppm stock solution of paraquat utilizing HILIC chromatography.

The last analyte, prodiamine, has been an interesting case in which by itself run as a standard, we are able to detect the analyte using our C18 chromatography method. However, when prodiamine is mixed with the other analytes the signal is lost. Figure 2.6 Prodiamine method development chromatograms, shows prodiamine standard by itself and in two other chromatograms of different complex mixtures. There are several possible explanations for this observation. The first is that prodiamine's signal may be getting suppressed when mixed with the other analytes such that while the mass spectrometer is scanning, there are too many scans taking place and prodiamine is not being detected. Another possibility is that prodiamine is interacting with other analytes

and effectively being neutralized or derivatized to some other compound that is not being targeted. Further development is underway that is investigating how prodiamine is affected by making a new set of mixtures that introduces a new analyte into the mixture in steps. Each mixture will be analyzed separately and at the same concentration in order to see how prodiamine interacts with each analyte.

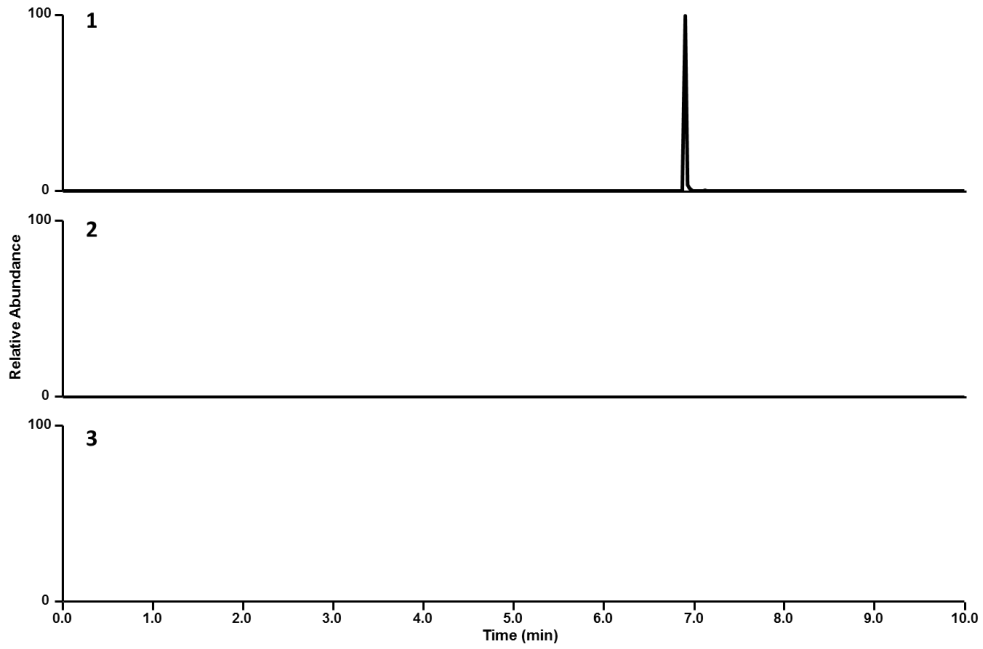


Figure 2.6. Prodiamine method development chromatograms. 1) A 10 ppb stock solution of prodiamine is successfully retained using a C18 column **2)** When prodiamine is mixed with all the other 13 analytes at a concentration of 10 ppb, signal is lost completely **3)** When prodiamine is mixed with another complex mixture at a concentration of 10 ppb containing mesotrione, simazine, atrazine, norflurazon, S-moc, fomesafen and prodiamine, signal is completely lost.

Extraction Process Method Development

The second main goal of this research project was the development of a new extraction methodology that would allow for a much more rapid approach for the analysis of agro chemicals in soil samples. Figure 2.7 Workflow diagram of QuEChERS extraction methodology, is a workflow diagram of the QuEChERS extraction procedure from soil preparation to analysis via UPLC-MS/MS as previously mentioned. This extraction method was applied to soil samples spiked with the 14 analytes of interest (Figure 2.1). Initial experiments were carried out with silt loam soil spiked with the pesticide mixture at a concentration of 5 parts per billion (ppb) (50 nanograms analyte per 10 grams of soil). This was a starting point for our eventual goal of detecting analytes at 1 ppb. Applying the reversed phase HPLC method optimized in the previous section to these soil extracts, it was possible to detect 10 of 14 analytes of interest, as shown in Table 2.2 Relative limit of quantitation levels for detectable analytes.

With the success of our method with silt loam, we shifted our focus to the development of the method for use on sandy loam and clay loam as well. As seen in Table 2.3 Average % relative standard deviation of three separate extractions performed for each type of soil at a 5 ppb controlled spike, when comparing all three types of soil, recoveries at a 5ppb spike are very strong. Recoveries were seen at greater than 86% for the seven analytes that were effectiveness of the method is lost with the clay loam. This is not surprising due to the potential interactions that clay presents with organic molecules. Clay tends to more strongly adsorb particles and also contains smaller pore sizes, making it harder to extract many analytes from this soil type [64].

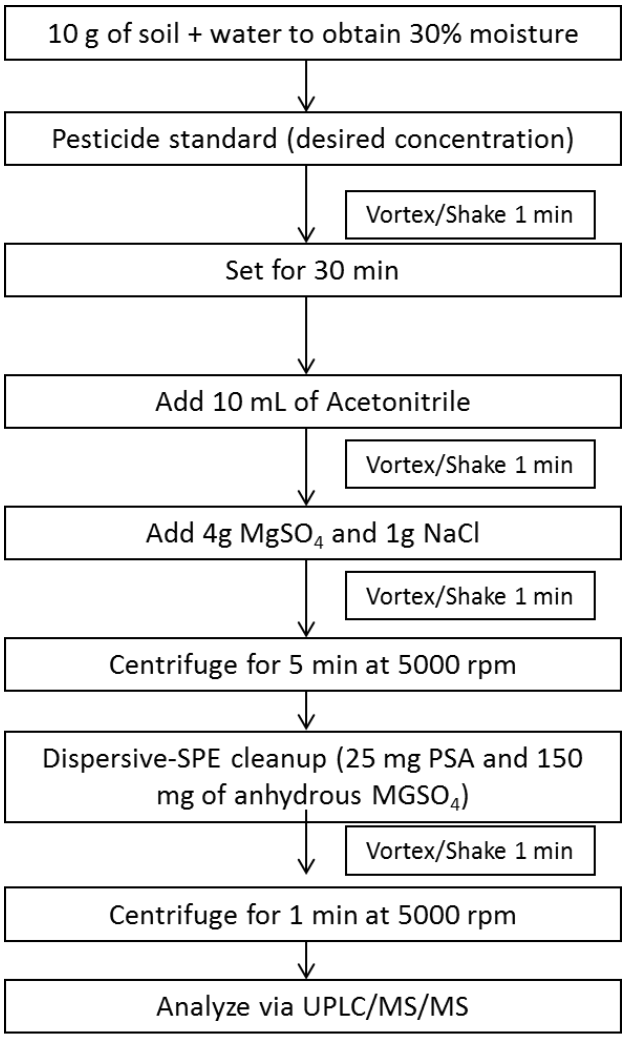


Figure 2.7. Workflow diagram of QuEChERS extraction methodology. This figure outlines our extraction procedure that is built off of the QuEChERS extraction methodology [42-44, 62].

Table 2.2. Relative limit of quantitation levels for detectable analytes. LOQ values represent the lowest concentration that achieves acceptable accuracy in back calculated concentration with a residual less than 15%. (-) indicates analyses currently in progress, data not yet available.

Pesticide	LOQ
Atrazine	2 ppb
Dicamba	5 ppb
Diquat	-
Fomesafen	7.5 ppb
Glyphosate	-
Mesotrione	2.5 ppb
Norflurazon	1 ppb
Paraquat	-
Primisulfuron	1 ppb
Prodiamine	-
Prosulfuron	1 ppb
Simazine	1 ppb
S-Metolachlor	1 ppb
Triasulfuron	2 ppb

Table 2.3. Average % relative standard deviation of three separate extractions performed for each type of soil at a 5 ppb controlled spike

Analyte	Silt (%)	Silt RSD	Sand (%)	Sand RSD	Clay (%)	Clay RSD
Atrazine[a]	-	-	-	-	-	-
Dicamba[a]	-	-	-	-	-	-
Fomesafen	96.1	7.9	94.2	8.2	86.9	0.4
Mesotrione[a]	-	-	-	-	-	-
Norflurazon	145.8	0.8	157.3	4.9	147.9	12.4
Primisulfuron	90.3	3.5	100.9	3.2	94.6	7.8
Prosulfuron	102.5	5.7	110.9	3.7	96.9	2.5
S-metolachlor	168.0	3.5	176.6	8.3	145.7	0.3
Simazine	105.7	6.3	100.3	8.1	121.1	5.7
Triasulfuron	99.6	8.0	102.2	5.9	82.9	2.1

[a] these analytes were not analyzed during this experiment.

Work is currently being performed looking at the effectiveness of the method at lower levels with clay loam, as well as analyzing the remaining three analytes of atrazine, dicamba, and mesotrione which have been detected previously.

One aspect of the proposed method that came in question was that of the final clean-up stage, the dispersive-SPE clean-up (Figure 2.7 Workflow diagram of QuEChERS extraction methodology). Specifically, it was of interest to determine the necessity of this step whether it could be eliminated from the procedure altogether, thus making the process more rapid and less expensive.

The original purpose of this clean-up stage was to reduce background interference from matrix components and facilitate more effective detection and quantitation of the analytes of interest. The common sorbent that is employed with this clean-up step is a primary-secondary amine (PSA) mixture. Initial tests with this sorbent yielded lower than expected results. This was particularly the case for negatively chargeable analytes (such as prosulfuron and fomesafen), which showed very low % recovery values with PSA adsorbent (Table 2.4 Data shown compares the use of a PSA-based dispersive-SPE cleanup sorbent and samples that did not undergo this cleanup step). It is likely that these analytes adsorbed to the PSA, forming ionic bonds with protonated amines. Table 2.5 Data shown compares the use of a PSA-based dispersive-SPE cleanup sorbent and samples that did not undergo this cleanup step, shows our extraction results comparing a separate C18 based dispersive-SPE sorbent, and the results from experiments that did not include the dispersive-SPE clean-up step.

Table 2.4. Data shown compares the use of a PSA-based dispersive-SPE cleanup sorbent and samples that did not undergo this cleanup step. It can be seen these negatively chargeable analytes are being strongly adsorbed to the cleanup sorbent, making the dispersive cleanup ineffective.

	Prosulfuron		Fomesafen	
	PSA	No Cleanup	PSA	No Cleanup
Silt	22	117	57	138
Sand	25	131	66	134
Clay	24	101	59	93

Table 2.5. Data shown compares the use of a C18 based dispersive-SPE cleanup sorbent and the results from eliminating the dispersive-SPE. Results are the average of three separate extractions using silt soil.

Analyte	C18 (%)	C18 RSD	No Cleanup (%)	No Cleanup RSD
Atrazine[a]	-	-	-	-
Dicamba[a]	-	-	-	-
Fomesafen	96.1	7.9	94.5	13.5
Mesotrione[a]	-	-	-	-
Norflurazon	145.8	0.8	141.8	9.3
Primisulfuron	90.3	3.5	88.6	4.4
Prosulfuron	102.5	5.7	97.9	9.4
S-metolachlor	168.0	3.5	159.8	7.6
Simazine	105.7	6.3	104.6	5.6
Triasulfuron	99.6	8.0	91.7	17

[a] indicates not analyzed in this experiment.

From these data, it was determined that there was not a need for the extra clean-up step in our methodology as we were easily able to detect and quantify our samples without it. There might still be other cases where adsorptive cleanup would be advantageous, however, such as a situation where a particular matrix caused high levels of interference, or if a very high matrix concentration was necessary to detect low levels of analyte.

Conclusion

Summary and Significance

This research project set out to develop a new analytical technique that would enable the simultaneous analysis of multiple agro chemicals from soil extracts. UPLC was successfully employed to separate 10 of the 14 analytes of interest. UPLC techniques offer excellent resolution and short run times, which allowed our method to be very rapid. Mass spectrometry with SRM as a detection method for the UPLC enabled us to identify and monitor two specific product ions from the precursor ion of interest. We were able to achieve limits of quantitation near the 1ppb level, with only a few of the analytes at slightly greater levels (up to 7.5ppb).

Our extraction procedure was based on QuEChERS technique. Using this technique, we were able to successfully apply this method to complex soil samples that allowed us to identify 10 analytes of interest simultaneously in a rapid fashion, achieving recovery levels close to 100% of a number of the analytes across three soil types (experiments to evaluate % recovery of the remaining analytes are ongoing). Our method

now allows for upwards of 10 samples to be extracted and prepped to be analyzed in an hour – a great increase in productivity compared to methods employed in the literature and by our colleagues at Syngenta Crop Protection, LLC.

This research project has shown that the QuEChERS methodology can successfully be utilized for the simultaneous analysis of complex agro chemical mixtures across several types of soil. Thus far, this methodology has limited use with soil due to the complex nature of soil. However, our research shows the effectiveness of a QuEChERS based protocol on soil, and the promise of applying this method to pesticides of diverse structural classes.

Future Directions

The method presented here shows great promise for updating and streamlining soil pesticide analysis. However, there is still work that needs to be done in order to optimize the method and move from controlled spiked samples to weathered field samples. An area of great interest is the ability of this method to be applied to clay soil. Our recoveries with the clay soil were sufficient at the 5ppb spiked level, but may not be the case at lower concentrations. Possible methods to enhance recovery from clay if problems arise include controlling pH levels during extraction with clay soil, as well as heating during the extraction process in the hopes of opening the soil pores to promote extraction of the analytes. After recovery optimization has been achieved, attention will be shifted to applying the method to the weathered field samples.

Method validation experiments are currently underway that will determine the effectiveness of our extraction protocol for the ten analytes mentioned previously.

Experiments will target 1ppb limits of quantification for each analyte, and it is still to be determined if concentrating samples is a necessary step before analysis via UPLC-MS to achieve these limits of quantification. Experiments will also be conducted to investigate any matrix suppression or enhancement that may be present in our analyses. Finally, experiments will also be conducted to determine the % recovery relative standard deviation from extraction to extraction at the LOQ and at ten times the LOQ.

Continuing efforts are also needed to develop the second analytical method utilizing HILC chromatography that will allow for the identification and quantitation of strongly polar analytes. Work has been done that suggests the effectiveness of this technique and experiments need to be continued in order to achieve effective separation. After optimization of the analytical technique has been completed, method validation studies can be done, similarly to that for the other method presented here.

REFERENCES

1. Attele, A.S.W., J. W.; Yuan, C., Ginseng Pharmacology. Biochemical Pharmacology, 1999. 58: p. 1685-1693.

2. Corbit, R.M.F., J. F. S.; Ebbs, S. D.; Murphy, L. L., Simplified Extraction of Ginsenosides from American Ginseng (Panax quinquefolius L.) for High-Performance Liquid Chromatography-Ultraviolet Analysis. Journal of Agricultural and Food Chemistry, 2005. 53: p. 9867-9873.

3. Cui, M.S., F.; Zhou, Y.; Liu, Z.; Liu, S., Rapid identification of saponins in plant extracts by electrospray ionization multi-stage tandem mass spectrometry and liquid chromatography/tandem mass spectrometry. Rapid Communications on Mass Spectrometry, 2000. 14: p. 1280-1286.

4. Jiang, Y.-S.J., Z-X; Umehara, H.; Ota, T., Cholesterol-dependent induction of dendrite formation by ginsenoside Rh2 in cultured melanoma cells. International Journal of Molecular Medicine 2010. 26: p. 787-793.

5. Lee, M.-J.C., J-S.; Cha, S-W.; Lee, K-S.; Lee, Z-W.; Hwang, G-S.; Lee, S-H.; Kamal, A. H. M.; Jung, Y-A.; Seung, N-S.; Woo, S-H., Variation in the ginsenoside profiles of cultivated ginseng (Panax ginseng C.A. Meyer) landraces in Korea. Process Biochemistry, 2011. 46: p. 258-264.

6. Lee, Y.J.J., Y. R.; Lim, W. C.; Park, W. K.; Cho, J. Y.; Jang, S.; Lee, S. K., Ginsenoside-Rb1 Acts as a Weak Phytoestrogen in MCF-7 Human Breast Cancer Cells. Arch Pharm Res, 2003. 26: p. 58-63.

7. Luchtefeld, R.K., E.; Smith, R. E., Determination of Ginsenoside Rb1, Rc, and Re in Different Dosage Forms of Ginseng by Negative Ion Electrospray Liquid Chromatography-Mass Spectrometry. Journal of Agricultural and Food Chemistry, 2004. 52: p. 4953-4956

8. Qi, L.-W.W., C-Z.; Yuan, C-S., Ginsenosides from American ginseng: Chemical and pharmacological diversity. Phytochemistry, 2011. 72: p. 689-699.

9. Sun, X.C., P.; Cook, S. L.; Jackson, G. P.; Harnly, J. M.; Harrington, P. B., Classification of Cultivation Locations of Panax quinquefolius L Samples using High Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry and Chemometric Analysis. Analytical Chemistry 2012. 84: p. 3628-3634.

10. Cho, J.P., W.; Lee, S.; Ahn, W.; Lee, Y., Ginsenoside-Rb1 from Panax ginseng C.A. Meyer Activates Estrogen Receptor-a and -b, Independent of Ligand Binding. The Journal of Clinical Endocrinology & Metabolism, 2004. 89: p. 3510-3515.

11. Liu, M.W., H.; Zhao, S.; Shi, X.; Zhang, Y.; Xu, H.; Wang, Y.; Li, X.; Zhang, L., Studies on target tissue distribution of ginsenosides and epidemium flavonoids in rats after intravenous administration of Jiweiling freeze-dried power. Biomedical Chromatography, 2011. 25: p. 1260-1272.

12. Wang, C.-Z.W., J. A.; McEntee, E.; Yuan, C-S., Saponins Composition in American Ginseng Leaf and Berry Assayed by High-Performance Liquid Chromatography. Journal of Agricultural and Food Chemistry, 2006. 54: p. 2261-2266.

13. Christensen, L.P.J., M.; Kidmose, U., Simultaneous Determination of Ginsenosides and Polyacetylenes in American Ginseng Root (Panax quinquefolium L.) by High-Performance Liquid Chromatography. Journal of Agricultural and Food Chemistry, 2006. 54: p. 8995-9003.

14. Fuzzati, N., Analysis methods of ginsenosides. Journal of Chromatography B, 2004. 812: p. 119-133.

15. Chan, T.W.D.B., P. P. H.; Cheng, S. W.; Kwok, I. M. Y.; Lau, F. W.; Xu, H. X. , Differentiation and Authentication of Panax ginseng, Panax quinquefolius, and Ginseng Products by Using HPLC/MS. Analytical Chemistry, 2000. 72: p. 1281-1287.

16. Ko, S.K.C., O. S.; Bae, H. M.; Im, B. O.; Lee, O-H.; Lee, B-Y., Quantitative Analysis of Ginsenosides Composition in Flower Buds of Various Ginseng Plants. J. Korean Soc. Appl. Biol. Chem. , 2011. 54: p. 154-157.

17. Li, W.F., J. F. , A validated method for quantitative determination of saponins in notoginseng (Panax notoginseng) using high-performance liquid chromatography with evaporative light-scattering detection. Journal of Pharmacy and Pharmacology 2001. 53: p. 1637-1643.

18. Liu, L.H., J.; Hu, X.; Li, K.; Sun, C. , Simultaneous determination of ginsenoside (G-Re, G-Rg1, G-Rg2, G-F1, G-Rh1) and protopanaxatriol in human plasma and urine by Lc-MS/MS and its application in a pharmacokinetics study of G-Re in volunteers. Journal of Chromatography B, 2011. 879: p. 2011-2017.

19. Qu, C.B., Y.; Jin, X.; Wang, Y.; Zhang, K.; You, J.; Zhang, H., Study on ginsenosides in different parts and ages of Panax quinquefolius L. Food Chemistry, 2009. 115: p. 340-346.

20. Wang, X.S., T.; Asafu-Adjaye, E.; Shiu, G. K., Determination of Ginsenosides in Plant Extracts from Panax ginseng and Panax Quinquefolius L. by LC/MS/MS. Analytical Chemistry, 1999. 71: p. 1579-1584.

21. Yu-chi, Z.Z.-f., P.; Chun-ming, L.; Feng-rui, S.; Zhi-qiang, L.; Shu-ying, L., Analysis of Low-polar Ginsenosides in Steamed Panax Ginseng at High-temperature by HPLC-ESI-MS/MS. Chem. Res. Chinese Universities 2012. 28: p. 31-36.

22. Popovich, D.G.H., C.; Durance, T. D.; Kitts, D. D., Retention of Ginsenosides in Dried Ginseng Root: Comparison of Drying Methods. Journal of Food Science, 2005. 70: p. 355-358.

23. Zhou W.; Li, J.L., X.; Yan, Q.; Zhou, P., Development and validation of a reversed-phase HPLC method for quantitative determination of ginsenosides Rb1, Rd, F2, and compound K during the process of biotransformation of ginsenoside Rb1. Journal of Separation Science, 2008. 31: p. 921-925.

24. Konsler, T.R.Z., S. W.; Shelton, J. E.; Staba, E. J., Lime and Phosphorous Effects on American Ginseng: II. Root and Leaf Ginsenoside Content and Their Relationship. J. Amer. Soc. Hort. Sci. , 1990. 115: p. 575-580.

25. van Breeman, R.B.H., C-R.; Lu, Z-Z; Rimando, A.; Fong, H. H. S.; Fitzloff, J. F., Electrospray Liquid Chromatography/Mass Spectrometry of Ginsenosides. Analytical Chemistry, 1995. 67: p. 3985-3989.

26. Ng, K.-M.C., C-M.; Wo, S-K.; Tam, P. K-H.; Lau, A. S-Y., Analytical application of acetate anion in negative electrospray ionization mass spectrometry for the analysis of triterpenoid saponins - ginsenosides. Rapid Communications on Mass Spectrometry 2006. 20: p. 1545-1550.

27. Yeo, C.-R.Y., C.; Wong, T-Y.; Popovich, D. G., A Quantified Ginseng (Panax ginseng C.A. Meyer) Extract Influences Lipid Acquisition and Increases Adiponectin Expression in 3T3-L1 Cells. Molecules 2011. 16: p. 477-492.

28. Ji, Q.C.H., M. R.; Henderson, G. L.; Gershwin, M. E.; Stern, J. S.; Hackman, R. M., Quantitative Determination of Ginsenosides by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Phytochemical Analysis, 2001. 12: p. 320-326.

29. Li, T.S.C.M., G.; Cottrell, A. C.; Gao, L., Ginsenosides in Roots and Leaves of American Ginseng. Journal of Agricultural and Food Chemistry, 1996. 44: p. 717-720.

30. Xia, C.W., G.; Sun, J.; Hao, H.; Xiong, Y.; Gu, S.; Shang, L.; Zheng, C., Simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma by liquid chromatography/electrospray ionization mass spectrometric method and its application to pharmacokinetic stody of 'SHENMAI' injection. Journal of Chromatography B, 2008. 862: p. 72-78.

31. Xie, J.-T.S., Z-H.; Hoek, T. L. V.; Chang, W-T.; Li, J.; Mehendale, S.; Wang, C-Z.; Hsu, C-W.; Becker, L. B.; Yin, J-J.; Yuan, C-S., Antioxidant effects of ginsenoside Re in cardiomyocytes. European Journal of Pharmacology, 2006. 532: p. 201-207.

32. Yu, J.-L.D., D-Q.; Chen, X-H.; Yang, H-Z.; Guo, N.; Cheng, G-F., Protopanaxatriol-Type Ginsenosides Differentially Modulate Type 1 and Type 2 Cytokines Production from Murine Splenocytes. Planta Medica, 2005. 71: p. 202-207.

33. Scott, G.I.C., P. B.; Ren, B. H.; Ren, J. , Ginsenosides Rb1 and Re decrease cardiac contraction in adult rat ventricular myocytes: role of nitric oxide. British Journal of Pharmacology 2001. 134: p. 1159-1165.

34. Huang, Y.-C.C., C-T.; Chen, S-C.; Lai, P-H.; Liang, H-C.; Chang, Y.; Yu, L-C.; Sung, H-W., A Natural Compound (Ginsenoside Re) Isolated from Panax ginseng as a Novel Angiogenic Agent for Tissue Regeneration. Pharmaceutical Research 2005. 22: p. 636-646.

35. Schlag, E.M.M.M.S., Ginsenoside content and variation among and within American ginseng (Panax quinquefolius L.) populations. Phytochemistry 2006. 67: p. 1510-1519.

36. Cech, R.A., Making Plant Medicine, ed. Williams2000: Horizon Herbs. 276.

37. Aktar, M.W., D. Sengupta, and A. Chowdhury, Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol, 2009. 2. 1-12.

38. Pimentel, D., Environmental and economic costs of the application of pesticides primarily in the United States. Environmenta, Development and Sustainability, 2005. 7: p. 229-252.

39. Arias-Estevez, M., et al., The mobility and degradation of pesticides in soils and the pollution of groundwater resources. Agric., Ecosyst. Environ., 2008. 247-260.

40. Caldas, S.S., et al., Evaluation of a Modified QuEChERS Extraction of Multiple Classes of Pesticides from a Rice Paddy Soil by LC-APCI-MS/MS. J. Agric. Food Chem., 2011. 59. 11918-11926.

41. Asensio-Ramos, M., et al., Evaluation of a modified QuEChERS method for the extraction of pesticides from agricultural, ornamental and forestal soils. Anal. Bioanal. Chem., 2010. 396. 2307-2319.

42. Correia-Sa, L., et al., Optimization of QuEChERS method for the analysis of organochlorine pesticides in soils with diverse organic matter. J. Sep. Sci., 2012. 35. 1521-1530.

43. Rashid, A., et al., Development of a simple extraction and clean-up procedure for determination of organochlorine pesticides in soil using gas chromatography-tandem mass spectrometry. J. Chromatogr., A, 2010. 1217. 2933-2939.

44. Anastassiades, M., et al., Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int., 2003. 86. 412-431.

45. Wilkowska, A. and M. Biziuk, Determination of pesticide residues in food matrices using the QuEChERS methodology. Food Chem., 2010. 125. 803-812.

46. Braganca, I., et al., QuEChERS: A new sample preparation approach for the determination of ibuprofen and its metabolites in soils. Sci. Total Environ., 2012. 433. 281-289.

47. Chen, L., C. Pan, and R.I. Krieger. Residue dynamics of procymidone in leeks and soil in greenhouses by smoke generator application. 2010. American Chemical Society.

48. Cunha, S.C. and J.O. Fernandes, Multipesticide residue analysis in maize combining acetonitrile-based extraction with dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry. J. Chromatogr., A, 2011. 1218. 7748-7757.

49. Park, K.H., et al., Development of QuEChERS-based extraction and liquid chromatography-tandem mass spectrometry method for quantifying flumethasone residues in beef muscle. Meat Sci., 2012. 92. 749-753.

50. Shi, C., et al., Determination of Oxadiargyl Residues in Environmental Samples and Rice Samples. Bull. Environ. Contam. Toxicol., 2010. 84. 236-239.

51. Yang, Q.-F. and Y.-L. Wu, Fast Determination of Fumonisin B1 and B2 in Corn Using a Modified QuEChERS Method and LC-MS-MS. Chromatographia, 2012. 75. 1075-1080.

52. Drozdzynski, D. and J. Kowalska, Rapid analysis of organic farming insecticides in soil and produce using ultra-performance liquid chromatography/tandem mass spectrometry. Anal. Bioanal. Chem., 2009. 394. 2241-2247.

53. Lesueur, C., et al., Comparison of four extraction methods for the analysis of 24 pesticides in soil samples with gas chromatography-mass spectrometry and liquid chromatography-ion trap-mass spectrometry. Talanta, 2008. 75. 284-293.

54. Padilla-Sanchez, J.A., et al., Application of a quick, easy, cheap, effective, rugged and safe-based method for the simultaneous extraction of chlorophenols, alkylphenols, nitrophenols and cresols in agricultural soils, analyzed by using gas chromatography-triple quadrupole-mass spectrometry/mass spectrometry. J. Chromatogr., A, 2010. 1217. 5724-5731.

55. Pinto, C.G., et al., Simplified QuEChERS approach for the extraction of chlorinated compounds from soil samples. Talanta, 2010. 81. 385-391.

56. Rouviere, F., et al., Multiresidue analysis of aromatic organochlorines in soil by gas chromatography-mass spectrometry and QuEChERS extraction based on water/dichloromethane partitioning. Comparison with accelerated solvent extraction. Talanta, 2012. 93. 336-344.

57. Fernandes, V.C., et al., Determination of Pesticides in Fruit and Fruit Juices by Chromatographic Methods. An Overview. J. Chromatogr. Sci., 2011. 49. 715-730.

58. Zhang, F., et al., Residue dynamics of pyraclostrobin in peanut and field soil by QuEChERS and LC-MS/MS. Ecotoxicol. Environ. Saf., 2012. 78. 116-122.

59. Lehotay, S.J., et al., Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection. J. AOAC Int., 2005. 88. 595-614.

60. Anagnostopoulos, C.J.B., G.; Miliadis, G. E. , Comparison of Liquid and Gas Chromatographic Mass Spectrometry for the Determination of Multiclass Pesticides at Low Concentrations. Spectroscopy Letters, 2012. 45: p. 202-218.

61. Harris, D.C., Quantitative Chemical Analysis. 8th Ed. ed. Vol. . 2010, New York: W. H. Freeman and Company.

62. Li, Y.D., F.; Liiu, X.; Xu, J.; Li, J.; Kong, Z.; Chen, X.; Song, W.; Wang, Y.; Zheng, Y., Simultaneous enantioselective determination of fenbuconazole and its main metabolites in soil and water by chiral liquid chromatography/tandem mass spectrometry. Journal of Chromatography A, 2011. 1218: p. 6667-6674.

63. Grumbach, E.S.F., K. J., Comprehensive Guide to HILIC2010, Milford, MA: Waters Corporation. 72.

64. Lagaly, G., Pesticide-clay interactions and formulations. Applied Clay Science, 2001. 18: p. 205-209.